U.S. Department of Agriculture
Animal and Plant Health Inspection Service
Wildlife Services

Blank Page Digitally Inserted

SEX DETERMINATION OF EAGLES, OWLS, AND HERONS BY ANALYZING PLASMA STEROID HORMONES

UNITED STATES DEPARTMENT OF THE INTERIOR
FISH AND WILDLIFE SERVICE
BUREAU OF SPORT FISHERIES AND WILDLIFE
Special Scientific Report—Wildlife No. 167

Blank Page Digitally Inserted

UNITED STATES DEPARTMENT OF THE INTERIOR
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

SEX DETERMINATION OF EAGLES, OWLS, AND HERONS BY ANALYZING PLASMA STEROID HORMONES

By

Michael P. Dieter

Patuxent Wildlife Research Center
Division of Wildlife Research
Bureau of Sport Fisheries and Wildlife
Laurel, Maryland 20810

Bureau of Sport Fisheries and Wildlife
Special Scientific Report--Wildlife No. 167
Washington, D.C. · May 1973

Blank Page Digitally Inserted

CONTENTS

ABSTRACT

Steroid hormone components from the plasma of several avian species, including bald eagles (Haliaeetus leucocephalus), barn owls (Tyto alba), and black-crowned night herons (Nycticorax nycticorax), were separated by centrifugal chromatography on silica gel columns. Identification of sex was primarily by staining of steroid hormone bands with iodine, charring with sulfuric acid, and observing fluorescence under ultraviolet light.

Techniques were standardized using plasma from known-sex Japanese quail (Coturnix coturnix japonica), mallard ducks (Anas platyrhynchos), and American kestrels (Falco sparverius). Before attempting to sex these species, or the eagles, owls, and herons that lack external sexual dimorphism, a "blind trial" was conducted on a known-sex pair of a particular species. All of these methodological tests were uniformly successful. Quantification and positive identification of each of the steroid hormone components have not been completed, but the described techniques are sufficient to distinguish sex.

INTRODUCTION

Several raptors including the bald eagle, golden eagle (Aquila chrysaetos), barn owl, and screech owl (Otus asia), and herons, such as the black-crowned night heron and Louisiana heron (Hydranassa tricolor), lack external sexual dimorphism both in the immature and mature state. Because these species occupy a terminal position in the food chain, and because some are particularly susceptible to the effects of environmental pollutants, they are currently being employed in experimental studies at the Patuxent Wildlife Research Center. It was necessary to develop a rapid and technically simple procedure to sex large numbers of these birds in order to form breeding pairs, and to gain a meaningful interpretation of subsequent experimental results. This preliminary report describes a method for the determination of sex by chromatographic separation of steroid hormones from the plasma, and identification of the components as products derived from either males or females.

PROCEDURES

Extraction and Concentration of Steroid Hormones

Heparinized syringes were used to draw blood from the heart, the alar vein, or the jugular vein, depending on the size of the bird. The plasma was separated and processed immediately, or the plasma frozen until used. Plasma was extracted three times with an equal volume of absolute chloroform plus one-twentieth volume of 1.0 N sodium hydroxide (Bush, 1961). Extracts were pooled in 10 ml Snyder tubes and taken down under nitrogen at 45°C to 100 µl.

Preparation of Chromatographic Columns

Except for minor differences outlined below, methods for slurry preparation, packing and developing of columns, and charring were essentially those outlined in the operator's manual supplied with a Centri-Chrom apparatus[1] (see also Ribi, et al. 1970). Eighty ml of a solution of ethyl acetate:cyclohexane (1:1 by volume) were added to 3 g of prepackaged silica adsorbent containing 6 percent water, then stoppered and stirred vigorously until homogeneous. Four ml of the silica slurry were added to 3 x 65 mm glass columns and packed for 7 min by centrifugation at 1500 G.

---

[1] Ivan Sorvall, Inc., Newtown, Conn.

Separation of Steroid Hormones on Chromatographic Columns

Of the 100 µl of chloroform extract, as much as possible was added directly to a reamed-out portion of the column, and to a porous stopper which rested on the top of the column. Three ml of the developing solvent (ethyl acetate:cyclohexane, 1:1) were added to the filling heads, and the chromatograms developed for 5 min by centrifugation at 2500 G. The ethyl acetate: cyclohexane system was selected from among twelve other solvent systems because it more readily separated steroids of the androstane series from those of the estrane series, and each of these from cholesterol (Randerath, 1963). The silica columns were extruded on trays and dried 10 min at 65°C.

Visualization of Steroid Hormones on Chromatographic Columns

Staining with iodine

Bands of steroid hormones were stained by exposing the chromatograms to iodine crystals in a closed container. The R_m values (distance of migration of steroid hormone bands from top of column) of unknown bands were compared to those of authentic steroid hormones. Further confirmation of the identity of individual steroid hormones was by sulfuric acid charring, viewing colors of bands in daylight, or fluorescence under ultraviolet light, or by elution and scanning of absorption peaks.

Charring with sulfuric acid

When charred, dried chromatograms were transferred to spatulas, inserted into the charring chamber, six drops of concentrated sulfuric acid added to the heating elements, and 90 volts applied until a satisfactory color had developed. The colors of individual bands were viewed in daylight. The charred chromatograms were also exposed to ultraviolet light, which caused certain steroid hormone bands to fluoresce.

Scanning of Absorption Peaks

When individual steroid hormones were to be scanned, cut bands with R_m values corresponding to steroid hormone standards were eluted with 2 ml of absolute chloroform, taken to complete dryness under nitrogen, and redissolved in 100 µl of chloroform. Fifty µl of this solution were added to 500 µl of concentrated sulfuric acid in a microcuvette. Sulfuric acid chromogen spectra over the range of 190 - 600 nm (Zaffaroni, 1953) were compared in eluted samples, using a Beckman Acta II, recording spectrophotometer.

Check of Recovery Using Radioactive Steroids

Percent recovery of ^{14}C-testosterone and ^{14}C-β-estradiol was estimated by radiochemical techniques. Known amounts of radioactive steroids were added to 5 ml plasma from male or female Japanese quail. Aliquots (0.2 ml) of each were removed, oxidized on a biological material oxidizer,[2] collected in

---

[2] Beckman Instruments, Inc., Silver Spring, Md.

Ready-Solv IX,[2] and counted in a liquid scintillation spectrometer.[3] Another 4 ml aliquot of each plasma sample was extracted as before, the steroids chromatographically separated, and iodine-stained bands with R_m values corresponding to authentic testosterone or β-estradiol cut off the columns. After oxidation the radioactivity in these bands, and that in the remaining portion of each column was measured.

RESULTS

Steroid Hormone Standards

Chromatographic separation and sensitivity of detection

Figure 1 illustrates the mobility of testosterone and β-estradiol when added to separate columns, and that of testosterone, β-estradiol, and cholesterol on the same column. Hormones were made up at a concentration of 1 µg/µl in chloroform:methanol (97:3) and 10 µg of each was normally added to the columns. In addition serial dilutions of testosterone and β-estradiol were added to the columns to check sensitivity; it was possible to detect the presence of 50 nanograms of these hormones with the iodine stain.

The R_m values of the steroid hormone bands stained with iodine were sufficiently distinct to easily separate testosterone (R_m of 27 mm) and β-estradiol (R_m of 34 mm) from each other, and both from cholesterol (R_m of 38 mm). In this solvent system, the R_m values of other steroids in the androstane series clustered around that of testosterone (R_m's of androstendione and dehydroisoandrosterone were 30 mm), and those in the estrane series clustered around β-estradiol (R_m's of progesterone and estrone were 32 and 36 mm). Charring with sulfuric acid resulted in a red color for the β-estradiol band, a blue color for the testosterone band, and a rust color for the cholesterol band.

Scanning of absorption peaks

The absorption peaks in the sulfuric acid chromogen spectra for testosterone and β-estradiol are shown in Figures 2 and 3, and were respectively, 285 - 290 nm, and 355 - 365 nm. These absorption peaks were found to be directly proportional to steroid hormone concentration up to 10 µg (fig. 4).

Figures 5 and 6 depict typical sulfuric acid chromogen spectra of eluates of standards of testosterone and β-estradiol, or after extraction of 4 ml of plasma from male and female, mature Japanese quail. The absorption maxima of the extracts were almost identical to those obtained when authentic steroids were added directly to the sulfuric acid and scanned, but peak heights were much less in magnitude.

---

[2] Beckman Instruments, Inc., Silver Spring, Md.
[3] Packard Instrument Co., Inc., LaGrange, Ill.

Percentage recovery of radioactive hormones added to plasma

The recovery of radioactive testosterone added to male plasma was 38.8 percent, and that of radioactive β-estradiol added to female plasma was 15.5 percent (table 1). Less than 0.2 percent ^{14}C-testosterone and 3.2 percent ^{14}C-β-estradiol was found on each of the remaining portions of the respective columns, indicating practically all of the testosterone or β-estradiol were localized in one band on each of the columns.

Steroid Hormone Unknowns

Avian species examined

Thus far these methods have been used to sucessfully distinguish the sexes of immature and mature bald eagles, mature barn owls, immature and mature black-crowned night herons, and a Louisiana heron. Prior to sexing a series of these species, a "blind trial" with known-sex pairs was conducted. Additional "blind trials" were conducted with known-sex pairs of mature Japanese quail, mature mallard ducks, and mature and immature American kestrels. In all of these trials, the determination of sex was correct.

Identification of steroid hormone components from males and females

Staining with iodine.-- In all instances chromatograms of extracts from male birds showed five distinct bands when stained with iodine, and those from females usually two, and sometimes three.

Charring with sulfuric acid.-- In extracts from birds identified as males, a band corresponding to authentic testosterone was visualized with iodine, but did not char with sulfuric acid. A band that chromatographed just above cholesterol and was not distinctly separated from it, charred a reddish-purple. In extracts from birds identified as females, a band corresponding to the β-estradiol standard was visualized with iodine, ran above cholesterol, and was distinctly separated from cholesterol; it charred a brilliant red like authentic β-estradiol. The most intensely stained band in both males and females corresponded to authentic cholesterol and charred a rust color like the standard.

Emission of fluorescence under ultraviolet light.-- When viewed under ultraviolet light after charring, the band corresponding to β-estradiol appeared red and cholesterol pink, but neither fluoresced. The reddish-purple band in extracts from males emitted a brilliant, blue fluorescence under ultraviolet light.

DISCUSSION

Recommendations for Analyses of Steroid Hormone Unknowns

The methods outlined for sex determination are very sensitive, relatively rapid, and require only a small plasma sample that may be frozen until analyzed. It is recommended that a known-sex pair of any given species always

be analyzed in a "blind trial" before analyzing a series of birds. This provides the investigator with an indication of any variation that may be encountered between species. It is mandatory that the plasma extract be concentrated, and that as much of the concentrated extract as possible be added to the silica gel column, in order to obtain enough steroid to be identified by visualization and charring procedures.

While uniform success was attained in distinguishing the sexes of all species examined, present techniques of extraction, separation, and elution must be improved before positive identification of individual steroid hormones can be obtained.

Authentication of Separatory Procedures

The recovery of testosterone (38.8 percent) was 2,3-fold greater than that for β-estradiol (15.5 percent). The very high lipid levels in the plasma of females resulted in emulsions when chloroform was added, and probably decreased the efficiency of the extraction procedures. Almost all of the radioactivity present on the columns resided in the bands corresponding to authentic testosterone or β-estradiol, indicating these steroid hormones separated well in the solvent system employed. This also shows that the R_m values for testosterone and β-estradiol are authentic and reproducible.

Circulating Steroid Hormones in Females

It appears that β-estradiol was present in all of the females of these species, since the R_m values, color after sulfuric acid charring, and appearance under ultraviolet light of the separated component, corresponded to that of authentic β-estradiol. However, Furr (see Gilbert, 1971) was reportedly unable to detect estrogens in the blood, and concluded their concentration must be less than 2 nanograms per ml. Gilbert (1971) cited others who found much higher concentrations, but cautioned that the methods may have been less specific. Blood levels of progesterone between 0.88 and 17.21 nanograms/ml were also reported (Gilbert, 1971). It is possible that the steroid band tentatively identified as β-estradiol in this study is in reality a combination of several steroids in the estrane series, since in the solvent system employed β-estradiol, estrone, and progesterone had similar R_m values (32, 34, and 36 mm). Cholesterol was present in both male and female extracts, and was always in the highest concentration of all the steroids detected.

Circulating Steroid Hormones in Males

The reddish-purple band in extracts from males emitted a brilliant blue fluorescence, but did not migrate with authentic testosterone; its identity is unknown. Another band that stained with iodine and did chromatograph with testosterone did not char with sulfuric acid. The plasma levels of testosterone reportedly ranged from 84 - 783 nanograms/100 ml in domestic fowl, less than 10 - 120 nanograms/100 ml in quail, 65 - 270 nanograms/100 ml in ducks, and 15 - 98 nanograms/100 ml in pigeons (Lake and Furr, 1971). Perhaps testosterone was the only steroid of the androstane series present in the species examined; if so, the levels extracted were evidently too low to be charred with sulfuric acid.

Scanning of Absorption Peaks

The elution of individual bands and scanning of absorption peaks were not particularly useful in regard to distinguishing sex, but in the future this procedure promises to provide a means of positive identification of individual steroid hormones in different species. Circulating levels of steroid sex hormones should rise during the breeding season (Gilbert, 1971; Lake and Furr, 1971), and with higher concentrations, pooled extracts may yield levels high enough to quantitate by comparison with absorption peaks of standards, or by fluorometric procedures.

ACKNOWLEDGMENTS

I wish to thank Dr. H. M. Ohlendorf, Dr. E. E. Klaas, Mr. S. N. Wiemeyer, and Dr. L. F. Stickel for permitting me to bleed their experimental birds. I am grateful to them and to Mr. J. R. Maestrelli and Mr. G. M. Sliker for helping me during the sampling procedures. I am also indebted to Dr. J. L. Ludke for conducting the radiochemical analyses.

REFERENCES

Bush, I. E.
1961. The chromatography of steroids. Pages 191-196. Pergamon Press, London. 437 p.

Gilbert, A. B.
1971. The endocrine ovary in reproduction. Pages 1449-1468 in D. J. Bell and B. M. Freeman, eds. Physiology and biochemistry of the domestic fowl. Vol. 3. Academic Press, London and New York. 1488 p.

Lake, P. E., and B. J. A. Furr.
1971. The endocrine testis in reproduction. Pages 1469-1488 in D. J. Bell and B. M. Freeman, eds. Physiology and biochemistry of the domestic fowl. Vol. 3. Academic Press, London and New York. 1488 p.

Randerath, K.
1963. Thin-layer chromatography. Academic Press, London and New York. 250 p.

Ribi, E., C. J. Filz, G. Goode, S. M. Strain, K. Yamamoto, S. C. Harris, and J. H. Simmons.
1970. Chromatographic separation of steroid hormones by centrifugation through columns of microparticulate silica. Journal of Chromatographic Science 8: 577-580.

Zaffaroni, A.
1953. Micromethods for the analysis of adrenocortical steroids. Recent Progress in Hormone Research 8: 51-83.

Table 1.--Recovery[a] of ^{14}C-testosterone and ^{14}C-β-estradiol added to plasma samples from male or female Japanese quail.

Samples	Total counts on column (CPM)	Total counts per sample (CPM)	Percent recovery
"testosterone" band	517,400	200,660	38.8
remainder of column		1,090	0.2
"β-estradiol" band	656,200	101,580	15.5
remainder of column		21,180	3.2

[a] Plasma samples were extracted with chloroform and chromatographed in ethyl acetate:cyclohexane (1:1 by volume). Separated bands were stained with iodine, cut off and oxidized, and counted in a liquid scintillation spectrometer. See "Procedures" for further details.

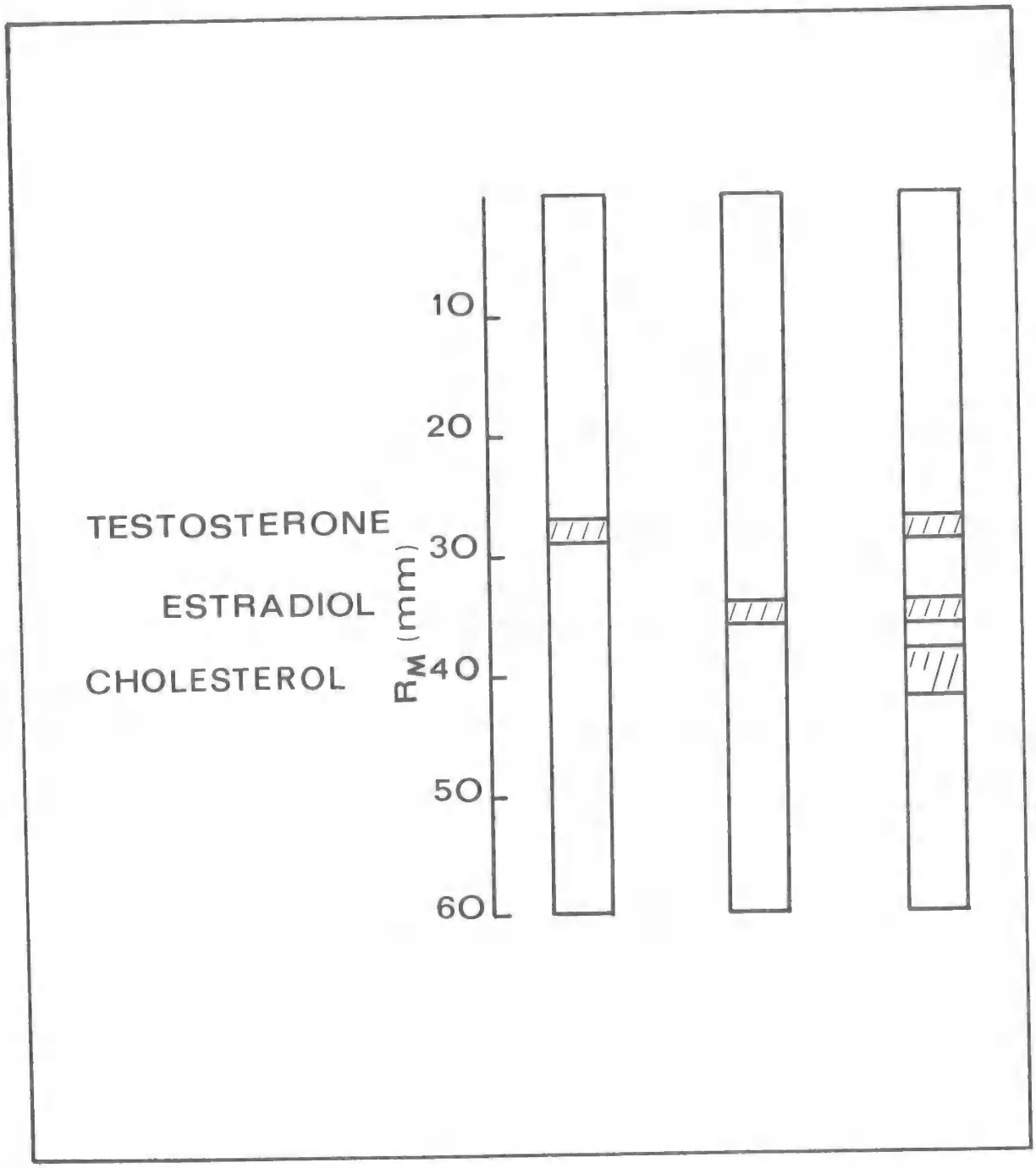

Figure 1.--Diagrammatic representation of migration rate (R_m) of authentic steroid hormones on silica gel columns. Columns contained 6 percent water and were packed for 5 min at 1500 G and developed for 7 min at 2500 G in an ethyl acetate:cyclohexane (1:1 by volume) solvent system. Steroid hormones were dissolved in chloroform:methanol (97:3 by volume) at a concentration of 1 µg/µl, and 10 µg of testosterone added to the first column, 10 µg of β-estradiol added to the second, and 10 µg of each, plus 10 µg of cholesterol added to the third column.

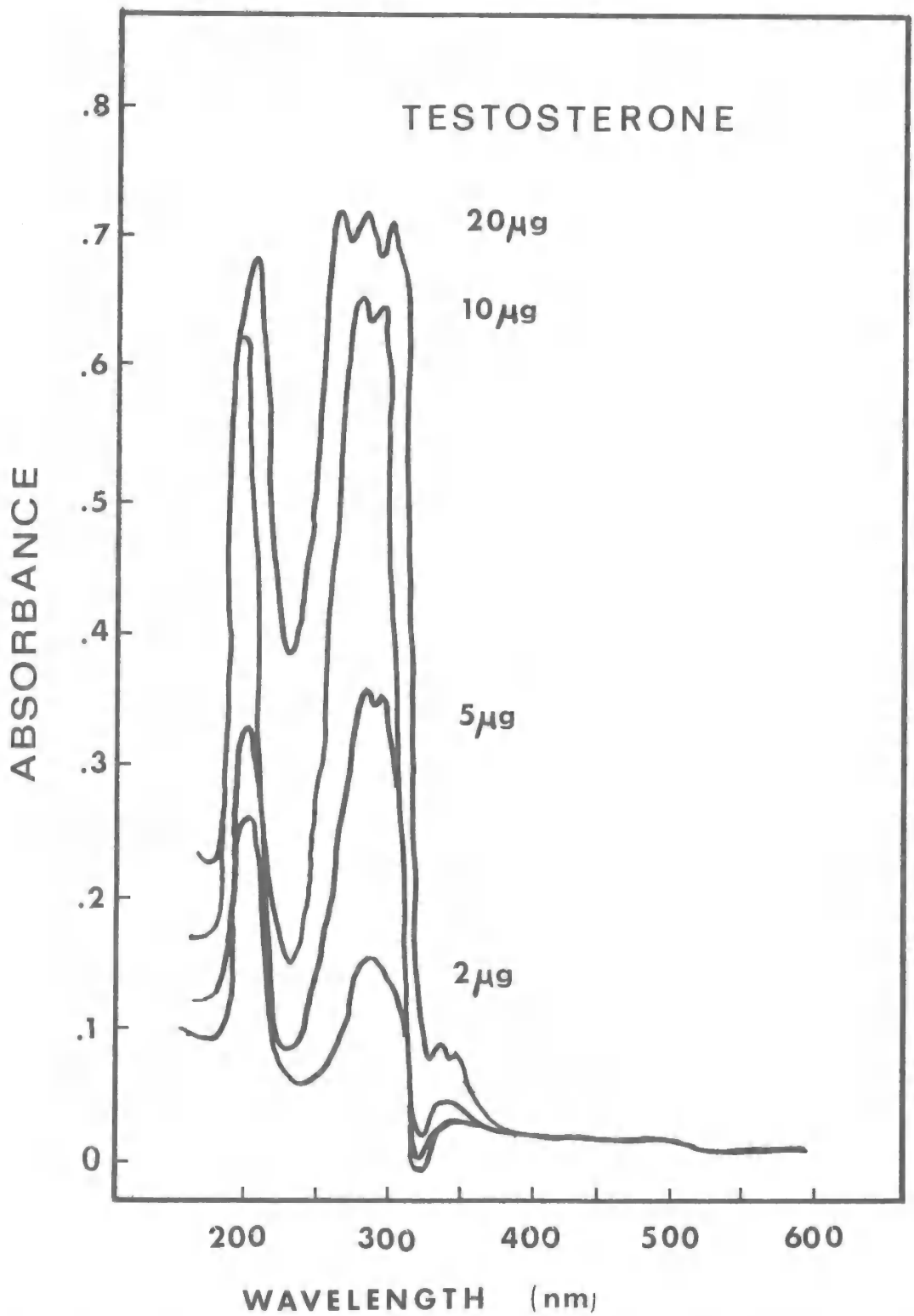

Figure 2.--Sulfuric acid chromogen spectra of varying amounts of authentic testosterone scanned from 190 - 600 nm at a speed of 100 nm/min and a chart speed of 2.54 cm/min. Full scale absorbance was set at 1.0.

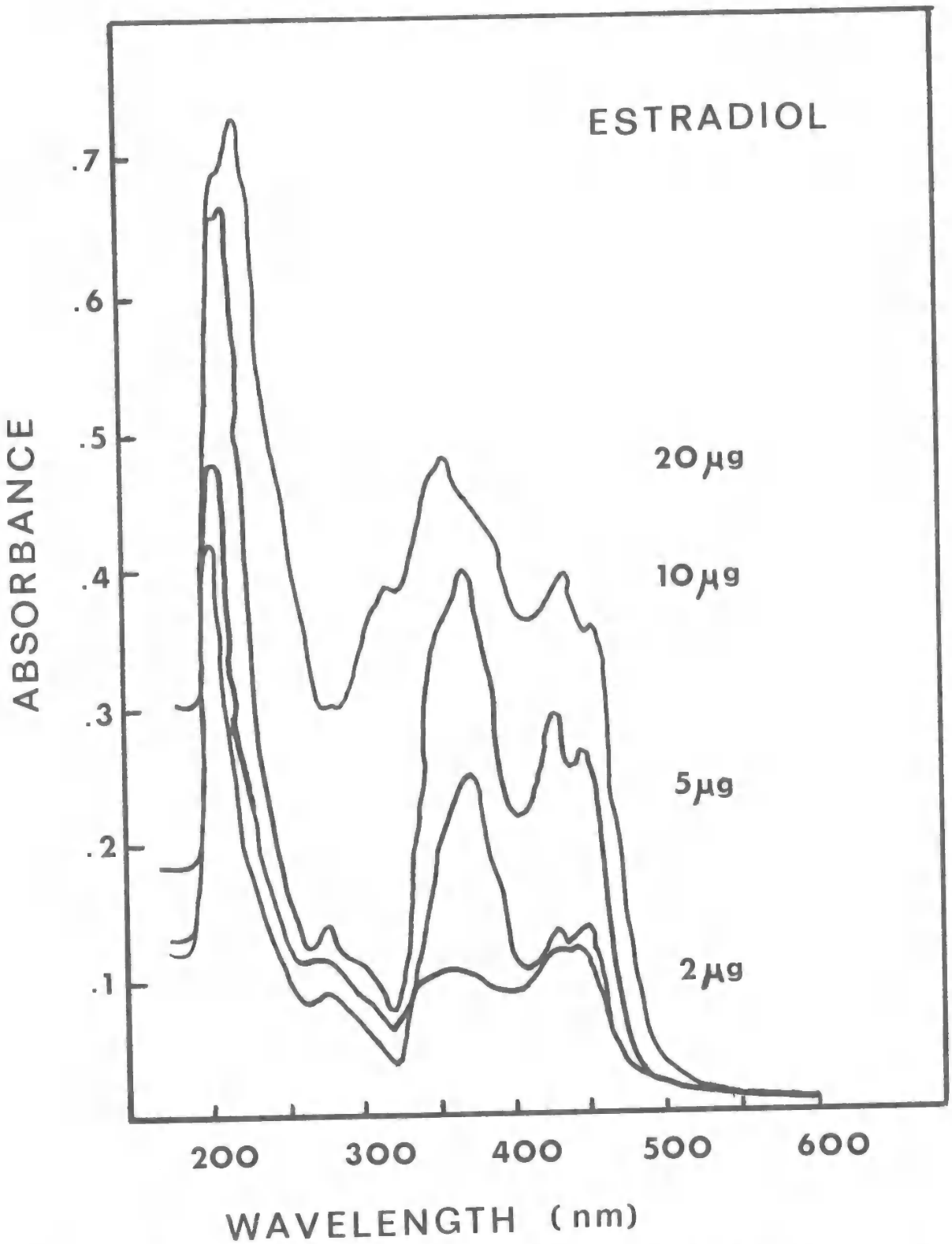

Figure 3.--Sulfuric acid chromogen spectra of varying amounts of authentic β-estradiol scanned from 190 - 600 nm at a speed of 100 nm/min and a chart speed of 2.54 cm/min. Full scale absorbance was set at 1.0.

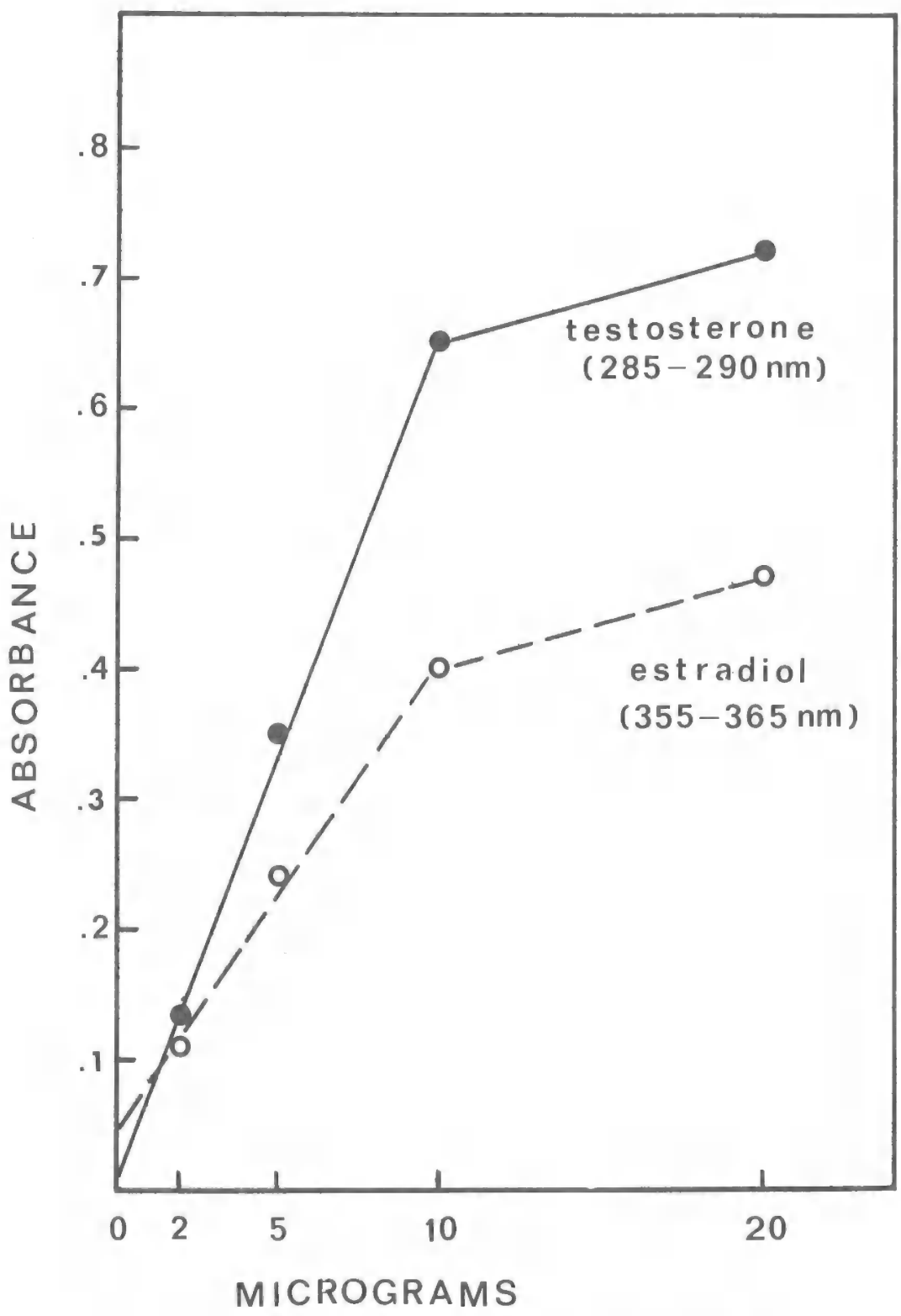

Figure 4.--A plot taken from Figures 2 and 3 illustrating the relationship between absorption peaks at 285 - 290 nm of testosterone, and those at 355 - 365 nm of β-estradiol, and the concentration of these steroid hormones.

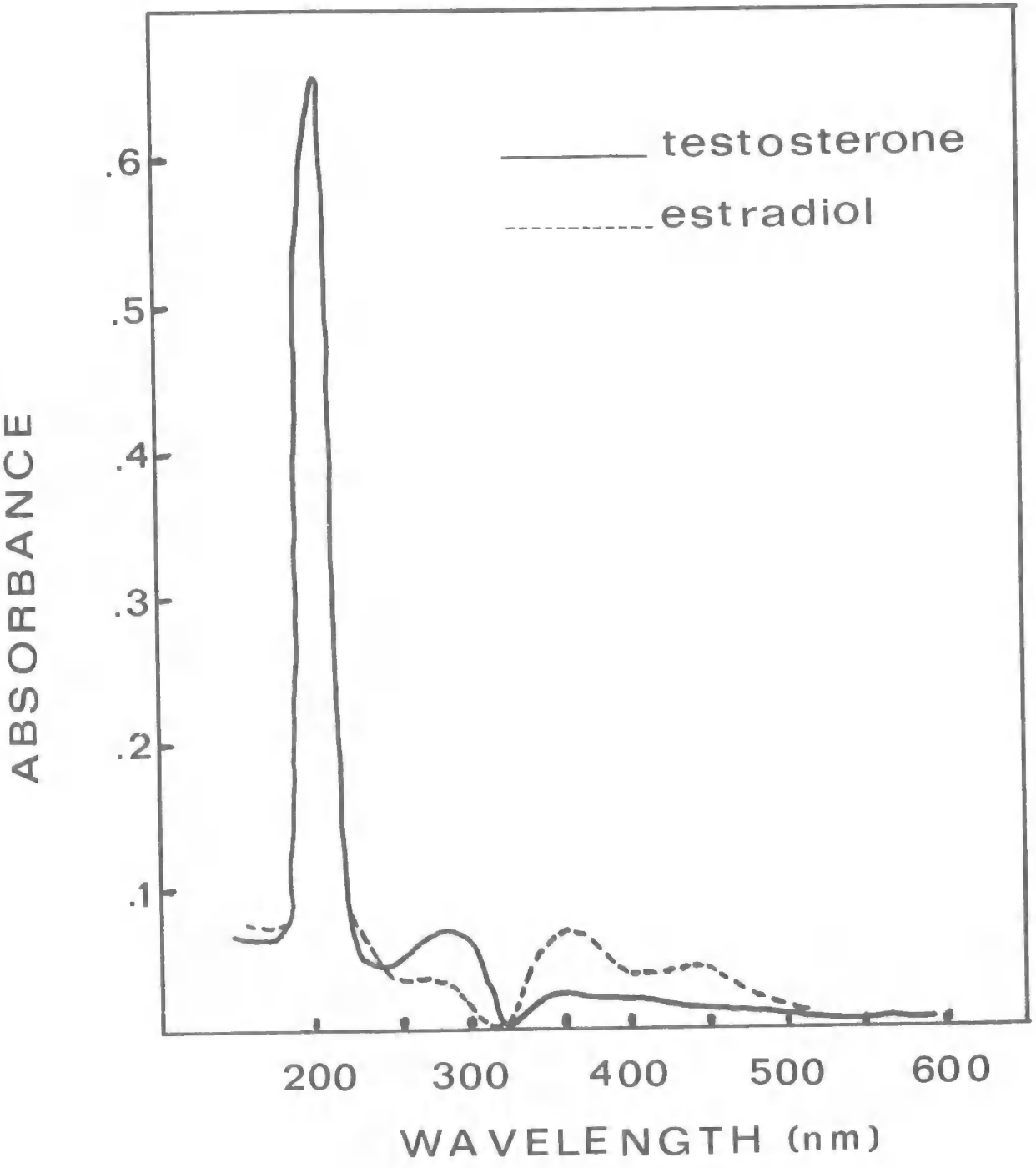

Figure 5.--Sulfuric acid chromogen spectra of authentic testosterone and β-estradiol separated and eluted from the same silica gel column. Solid line depicts scan of testosterone and dashed line that of β-estradiol.

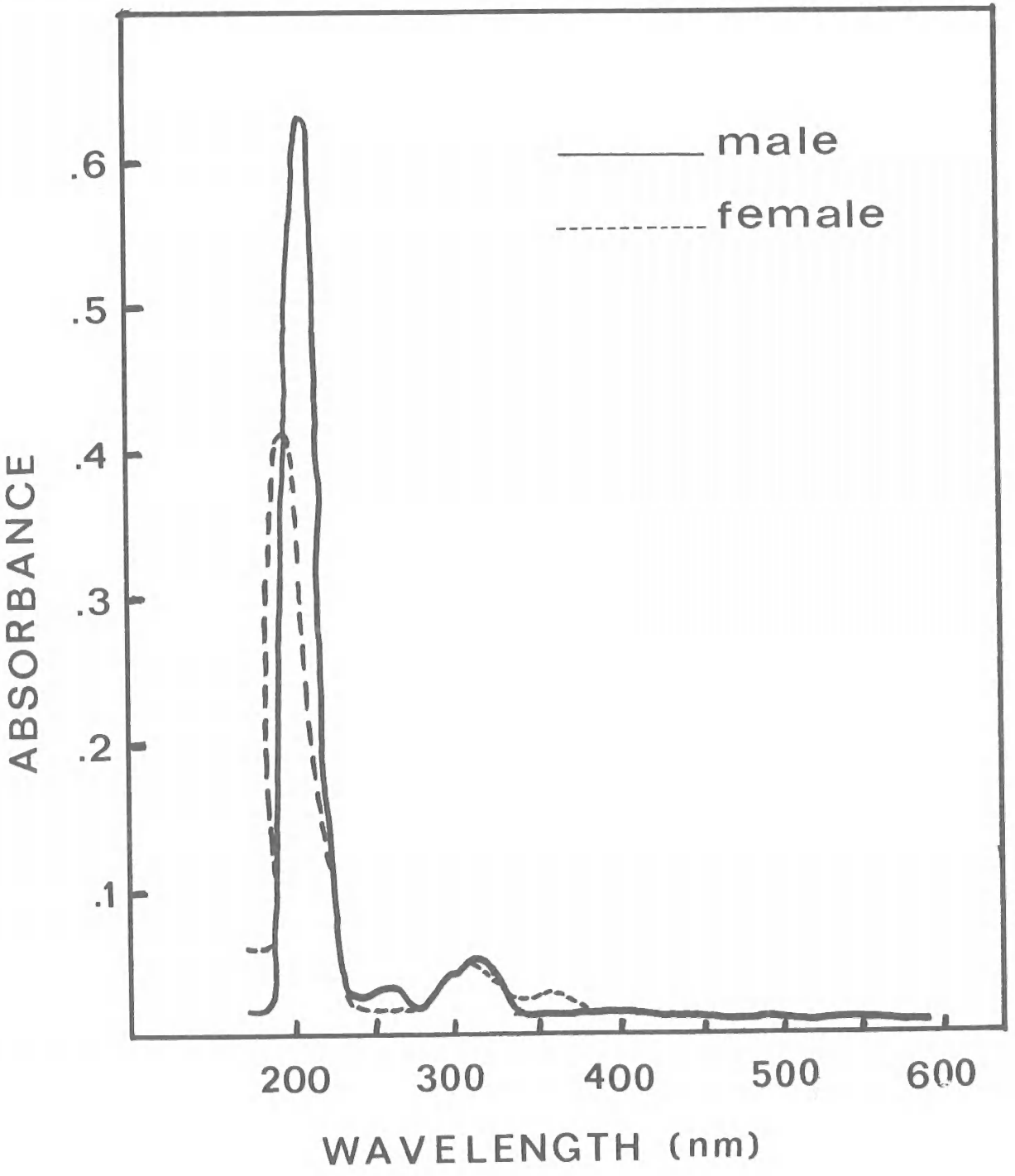

Figure 6.--Sulfuric acid chromogen spectra of extracts from male and female Japanese quail. Solid line depicts scan of eluate from male extract chromatographing with authentic testosterone, and dashed line that of eluate from female extract chromatographing with authentic β-estradiol.

Blank Page Digitally Inserted

SERIAL F&WS SSR-W no. 167
Dieter, Michael P.
Sex determination of eagles, owls, and herons by analyzing plasma steroid hormones 90017835

As the Nation's principal conservation agency, the Department of the Interior has basic responsibilities for water, fish, wildlife, mineral, land, park, and recreational resources. Indian and Territorial affairs are other major concerns of this department of natural resources.

The Department works to assure the wisest choice in managing all our resources so that each shall make its full contribution to a better United States now and in the future.

UNITED STATES
DEPARTMENT OF THE INTERIOR
FISH AND WILDLIFE SERVICE
BUREAU OF SPORT FISHERIES AND WILDLIFE
WASHINGTON, D. C. 20240

POSTAGE AND FEES PAID
U.S. DEPARTMENT OF THE INTERIOR
INT 423

U.S.MAIL